PATENT
0020-4491P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Kyogo ITOH et al

Serial Number: 09/202,047 Group: 1642
Filed: December 7, 1998 Examiner: Sheela Huff

For: Tumor Antigen Proteins, Genes Therefor, and Tumor Antigen Peptides

DECLARATION UNDER 37 C.F.R. § 1.132

Dear Sir:

The undersigned, Kyogo Itoh, M.D., Ph.D., hereby declares and states as follows:

1. My Curriculum Vitae including a list of my publications and credentials, is attached hereto as Appendix A.

2. On the basis of the qualifications set forth in my Curriculum Vitae, I submit that I am an expert in the fields of molecular biology and oncology. I have long been engaged in research on tumor antigens and identified several novel tumor antigens such as SART-2, SART-3, Cyclophilin B, and ART-1 in addition to SART-1 as the subject matter of the present invention.

3. I have studied the Office Action issued on February 1, 2001 and understood what is the essence of the Office Action.

4. I am the primary inventor of the US Patent Application number 09/202,047 (hereinafter, "present application") and am familiar with the scientific and practical significance of the invention. I am also familiar with cited Nakao et al,

Cancer Res. 55: 4248-4252, October 1, 1995 (hereinafter, "Nakao et al") as one of co-authors.

5. In my capacity as set forth above, I would like to express my opinion on the Office Action especially on the following issues:

1) Claims 7 and 8 meets the requirements under 35 USC § 112; and
2) Claims 6-9 and 12-13 are novel over Nakao et al

5.1. Claims 7 and 8 meets the Disclosure Requirements

5.1.1. The amended claims 7 and 8 read:

Claim 7: *An isolated tumor antigen peptide consisting of part of the protein of claim 6, which binds to MHC class I antigen to be recognized by CTLs.*

Claim 8: *An isolated tumor antigen peptide of claim 7 which comprises the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence of SEQ ID NO: 1.*

The Examiner stated as follows:

"The specification fails to enable any peptides that are produced through "intracellular decomposition" which bind MHC class I antigen and can be recognized by T cells..."

However, one of ordinary skill in the art could make and use the peptides as claimed in claims 7 and 8 above on the basis of the description, teaching, and guidance in the specification in view of the technical background at the filing date of the priority application of the present application (June 7, 1996, hereinafter, "priority date").

5.1.2. The present invention is based on the successful cloning of a novel tumor antigen protein, which is now called "SART-1". Before the priority date, there had

been no tumor antigens identified other than those originated from a special tumor, i.e., melanoma. Accordingly, it was very much desired a tumor antigen protein to be isolated from more common tumors such as squamous cell carcinoma (see, page 7, line 1 through page 8, line 22 of the specification). The present inventors, for the first time, succeeded in the cloning and the characterization of a novel tumor antigen protein (SART-1) from a tumor other than melanoma, that is, squamous cell carcinoma derived from esophageal cancer. J. Exp. Med., one of most famous learned journals, published our research results regarding cloning and characterization of SART-1 in recognition of the significance. See, Appendix B (*J. Exp. Med.* **187**: 277-288, 1998).

5.1.3. The cloning of SART-1 was extremely difficult. In the absence of any genetic information, we, the inventors, had to use the expression cloning technique, which comprises expressing randomly an enormous numbers of cDNA clones, determining the activity of each clone, and selecting the intended clone(s). We repeated the procedures again and again and finally isolated one cDNA clone from squamous cell carcinoma derived from esophageal cancer, as described in Examples 1 to 3 of the present application. The clone is referred to as "K3" in the present specification and now known as "SART-1". We then confirmed that the gene encoding SART-1 (K3), when transformed into a cell, yields a peptide fragment(s) through the intracellular processing of SART-1, which fragment binds to major histocompatibility complex (MHC) class I antigen, i.e., HLA antigen, and is recognized by cytotoxic T lymphocytes (CTLs), as recited in claim 6. These results are shown in Table 2 in Example 3 of the present specification. In Table 2, the activity of SART-1 (K3) is determined by measuring the amount of IFN-γ generated by CTLs. It is known that CTL produces IFN-γ only when it recognizes a peptide presented by MHC class I antigen (HLA antigen), which peptide have been generated through the intracellular processing, and hence the results in Table 2 certainly prove that "an isolated protein" of the present invention as defined in claim 6 has the activity as recited in the said claim.

5.1.4. As mentioned above, the cloning of SART-1 could not be achieved without resolving considerable difficulties. Once the tumor antigen protein was cloned and identified, the next step for determining a part (portion) thereof having an

activity as a tumor antigen peptide could be carried out by a technique known in the art.

5.1.5. It was commonly known in the art that antigen peptides which are bound and presented by MHC class I antigen (HLA antigen) are generally 8-12 amino acid long. For example, antigen peptides listed in *Immunogenetics*, **41**:178-228, 1995 are all have amino acid sequences of 8 to 12 amino acids. See, Table. Likewise, *J. Immunol.*, **152**:3913, 1994 (see, abstract) and *J. Immunol.*, **152**:3904, 1994 also describes the requisite amino acid length of antigen peptide presented by MHC class I antigen (HLA antigen) to be 8-12 a.a. and 8-11 a.a., respectively. These publications have been filed with the USPTO as IDS.

5.1.6. Further, it was also known that, regarding some MHC class I antigens such as HLA-A24, there is a rule (motif) in the amino acid sequence of a peptide to be presented thereby. Accordingly, one could even identify a tumor antigen peptide based on a peptide sequence comprising the motif. The state of art at the priority date is described in the above-mentioned three documents in detail, as well as in the present specification (see, page 16, lines 16-23). By means of such a method, we identified peptide "690-698" as an antigen peptide, which is described in Example 4 at page 37, lines 12-15 of the present specification.

5.1.7. With regard to a tumor antigen peptide restricted to HLA antigen lacking "rule" in the amino acid sequence, it could be identified according to the method described in Example 4 of the present specification. That is, from a cDNA encoding a tumor antigen protein, clones of various length were prepared using Deletion Kit and analyzed the activity by measuring the amount of IFN-γ produced by CTLs as mentioned above. Peptide fragments of 8-12 amino acids were then synthesized based on the sequence of a clone having activity, and allowed to contact with cells for assay to determine whether of not the synthetic peptide fragments have the activity of binding to HLA antigen and being recognized by CTLs, as claimed in claim 7. After the series of procedures, there obtained three tumor antigen peptides corresponding to amino acid sequences "736-744", "749-757" and "785-793". One ordinary skilled in the art could identify any tumor antigen peptides by following the procedures described in

Example 4 involving "deletion".

5.1.8. As mentioned above, once a "tumor antigen protein" has been identified, one ordinary skilled in the art could identify tumor antigen peptides as recited in claims 7 and 8 on the basis of the known techniques regarding the length and motif of peptides capable of binding to MHC class I antigen, guidance and working examples in the present specification. Accordingly, the peptides as the subject matters of claims 7 and 8 are described in the specification to such an extent to enable one ordinary skilled in the art to make and use the same.

5.2. Claims 6-9 and 12-13 are novel over Nakao et al

5.2.1. I am familiar with the cited Nakao et al document, which reports the results of research work conducted in my office under my supervision. The results are those obtained prior to the cloning or identification of SART-1. It is my conclusion that Nakao et al did not anticipate the tumor antigen peptide derived from SART-1 on the basis of the facts below.

5.2.2. The Examiner takes the position that the tumor antigen peptide of the present invention and the antigen peptide of Nakao et al are identical because the both peptides are originated from KE-4 and recognized by KE-4CTL; however, this is not pertinent. That is, there are many tumor antigens which are distinct from SART-1 and yet expressed on KE-4 and recognized by KE-4CTL. We have so far identified such tumor antigens, namely, SART-2 (Nakao, M. et al, *J. Immunol.*,**164**, 2565 (2000)) and SART-3 (Yang, D. et al, *Cancer Res.*,**59**: 4056(1999)), which are distinct from each other and also from SART-1 in terms of structure.

5.2.3. Further, the subject matter as claimed in the present application is "isolated tumor antigen protein" and "isolated tumor antigen peptide", while Nakao et al do not describe such an "isolated" protein or peptide. Examiner pointed out that Nakao et al teach a peptide antigen expressed on SCC from KE-4 tumor cells specifically making reference to Fig. 3 on page 4251. Although the said Fig. 3 indicates the presence of a substance having activity in fraction No. 23, there are no evidences showing that the said fraction contains a single

protein/peptide, much less SART-1 of the present invention.

5.2.4. It is well known in the art that HPLC, when conducted under the conditions set forth in Nakao et al, gives peaks each containing plural peptides. Actually, it was technically very difficult for us to isolate and identify tumor antigen protein or peptide from the active fraction No. 23. We then used the "expression cloning technique" and succeed in the isolation and identification of SART-1 after continuous endeavors. It would be objectively understood that the active peak (No. 23) corresponds to a mixture of peptides of unknown structure, which is clearly distinct from the isolated tumor antigen protein or peptide of the present invention.

6. In summary, it is my conclusion that the peptides as the subject matters of claims 7 and 8 are described in the specification to such an extent to enable one ordinary skilled in the art to make and use the same, and that, on June 7, 1996, Nakao et al did not anticipate the tumor antigen peptide derived from SART-1.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: June 6, 2001

Kyogo Itoh, M.D., Ph.D.

CURRICULUM VITAE

Kyogo Itoh, M.D., Ph.D.

Title and Affiliation:

Primary appointment: Professor and Chairman, Department of Immunology, Director of Kurume University Research Center for Innovative Cancer Therapy, Kurume University School of Medicine, Kurume, Fukuoka 830, Japan.

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Education:

Tohoku Univ. School of Medicine, Sendai, Japan, Ph.D. 1981, Immunology

Hirosaki Univ. School of Medicine, Hirosaki, Japan, M.D., 1974, Medicine.

Academic and Professional Appointments:

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1995-present, Adjunct Professor, Department of Immunology, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas, U.S.A.

1992-1995, Adjunct Associate Professor, Departments of Immunology and General Surgery, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas, U.S.A.

1990-1992, Associate Immunologist and Associate Professor of Immunology (Tenure),

Department of Immunology (General Surgery), and Chief of the TIL Core Lab, The University of Texas, M.D. Anderson Cancer Center, Houston, TX.

1990, Assistant Immunologist and Assistant Professor, Department of Immunology (General Surgery), and Chief of the TIL Core Lab, The University of Texas M. D. Anderson Cancer Center, Houston, TX.

1987-1990 Assistant Immunologist and Assistant Professor, Department of General Surgery (Immunology) and Chief, TIL Core Lab, The University of Texas M. D. Anderson Cancer Center, Houston, TX.

1987-1992 Member of the Graduate Faculty, Graduate School of Biomedical Sciences, The University of Texas Health Science Center at Houston, Houston, TX.

1986-87 Visiting Scientist, Department of General Surgery (Immunology), The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX.

1985-86 Postdoctoral Fellow, Department of General Surgery (Immunology), The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX.

1984-85 Research Associate, Departments of Surgery and Immunology, The University of Alabama at Birmingham, Alabama.

1978-88 Senior Instructor, Department of Microbiology, Tohoku University School of Dentistry and Medicine, Sendai, Japan.

1974-78 Surgeon, The Second Department of Surgery, Hirosaki University School of Medicine, Hirosaki, Japan.

1974-75 Surgeon, Division of Surgery, Misawa Central Hospital, Misawa, Japan.

Editorships and Editorial Board Memberships

Associate editor for J. Immunotherapy (1997-)

A member of editorial board for International Journal Clinical oncology(1996-)

Society Memberships:

The American Association of Immunologists (1987-present)
The American Association for Cancer Research (1988-present)
The American Society of Clinical Oncology (1990-present)
The Japanese Society for Immunology (1978-1984; 1992-present)
The Japanese Cancer Society (1979-1984; 1992-present)
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Bibliography

Kyogo Itoh, M.D., Ph.D.

a. Published and accepted articles in refereed journals:

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NK and noncytotoxic T cells in RCC. J. Immunother., *10:* 313-325, 1991.

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b. Invited Articles in Journals, and Book chapters (written by English only):

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c. Others

1. Institutional Service Responsibilities and Other Duties:

Chief, TIL Core Laboratory, Smith Research Building, Houston, Texas, 1988 - 1992.

Committee Member, Institutional Animal Care and Use Committee 1991-92.

A Gene Encoding Antigenic Peptides of Human Squamous Cell Carcinoma Recognized by Cytotoxic T Lymphocytes

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Summary

Except for melanomas, tumor antigens recognized by cytotoxic T lymphocytes (CTLs) are yet unidentified. We have identified a gene encoding antigenic peptides of human squamous cell carcinomas (SCCs) recognized by human histocompatibility leukocyte antigens (HLA)-A2601–restricted CTLs. This gene showed no similarity to known sequences, and encoded two (125- and 43-kilodalton [kD]) proteins. The 125-kD protein with the leucine zipper motif was expressed in the nucleus of the majority of proliferating cells tested, including normal and malignant cells. The 43-kD protein was expressed in the cytosol of most SCCs from various organs and half of lung adenocarcinomas, but was not expressed in other cancers nor in a panel of normal tissues. The three nonapeptides shared by the two proteins were recognized by the KE4 CTLs, and one of the peptides induced in vitro from peripheral blood mononuclear cells (PBMCs) the CTLs restricted to the autologous tumor cells. The 43-kD protein and this nonapeptide (KGSGKMKTE) may be useful for the specific immunotherapy of HLA-A2601$^+$ epithelial cancer patients.

Many genes encoding tumor-rejection antigens recognized by CTLs were identified from cDNA of melanomas (1–6). Further, a large number of nonapeptides recognized by HLA class I–restricted CTLs cytotoxic to melanoma cells have been identified in the past five years (5–15). Some of them are under clinical trials as cancer vaccines, and major tumor regression in several HLA-A1$^+$ melanoma patients was reported in the vaccine therapy with the melanoma antigen (MAGE)-3 peptide (16). Therefore, these nonapeptides recognized by the CTLs could be a new tool for the specific immunotherapy of melanoma. However, no peptides are yet identified from human squamous cell carcinomas (SCCs)[1] one of the major human cancers, except for a mutated CASP-8 (17). We previously reported the HLA-A2601–restricted and tumor-specific CTL line recognizing peptide antigen(s) expressed on SCCs (18). In this study, we have investigated a gene encoding tumor antigen recognized by this CTL line, and report a new gene encoding two novel proteins and three nonapeptides as the antigens recognized by the HLA-A2601–restricted CTLs.

Materials and Methods

Identification of 6A1-1D7 Genes. Expression-gene cloning methods developed by T. Boon and colleagues (4, 6) were used in this study to identify a gene coding tumor antigen recognized by the KE4 CTLs (18). In brief, messenger RNA (mRNA) of the KE4 tumor cells was converted to cDNA, ligated to SalI adapter, and inserted into the expression vector pSV-SPORT-1 (GIBCO BRL, Gaithersburg, MD). cDNA of HLA-A2601 or HLA-A0201 was obtained by reverse transcription PCR (RT-PCR), and was cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). Both 200 ng of plasmid DNA pools or clones of the KE4 cDNA library and 200 ng of the HLA-A2601 cDNA were mixed with 1 μl of lipofectin in 70 μl of OPTI-MEM® (GIBCO BRL) for 15 min. 30 μl of the mixture was then added to the VA13 (2×10^4) cells and incubated for 5 h. 200 μl of the RPMI-1640 medium containing 20% FCS was then added and cultured for 2 d followed by adding the KE4 CTLs (10^4 cells/well; reference 8). After a 18-h incubation, 100 μl of supernatant was collected to measure IFN-γ by an ELISA kit (Otsuka Pharm. Co., Tokyo, Japan) in a duplicate assay. DNA sequencing was performed with dideoxynucleotide sequencing method using DNA Sequencing kit (Perkin-Elmer Corp., Foster, CA) and analyzed by ABI PRISM™ 377 DNA Sequencer (Perkin-Elmer).

[1] *Abbreviations used in this paper:* aa, amino acid; gp, glycoprotein; GST, glutathione S-transferase; LAP, liver-enriched transcriptional-activator protein; LIP, liver-enriched transcriptional-inhibitory protein; mRNA, messenger RNA; nt, nucleotide; ORF, open reading frame; S-D, Shine-Dalgarno; SART, squamous cell carcinoma antigen recognized by T cells; SCC, squamous cell carcinoma.

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Northern Blot Analysis. Nylon membranes (Hybond-N^+; Amersham, Buckinghamshire, UK) with UV-fixed total RNAs (5 μg/lane) extracted from the various cells or UV-fixed poly A^+ RNA (2 μg/lane) from various tissues were prehybridized for 10 min and hybridized overnight at 65°C in the same solution (7% SDS, 1 mM EDTA, 0.5 M NaH_2PO_4, pH 7.2) containing ^{32}P-labeled *6A1-1D7* probe. The membranes were washed three times at 65°C in a washing buffer (1% SDS, 40 mM Na_2HPO_4, pH 7.2), and then autoradiographed. The relative expression level of the squamous cell carcinoma antigen recognized by T cells 1 (*SART-1*) mRNA was calculated by the following formula: index = (*SART-1* density of a sample/β-actin density of a sample) × (β-actin density of the KE4 tumor/*SART-1* density of the KE4 tumor).

Cloning of the SART-1 Gene. We tentatively named this gene encoding a tumor antigen recognized by the KE4 CTLs as the *SART-1* gene. The *SART-1* clone was obtained from both PBMCs (SuperScript™ Human Leukocyte cDNA Library in pCMV-SPORT; GIBCO BRL) and KE4 cDNA libraries by the standard colony hybridization method with the ^{32}P-labeled *6A1-1D7* cDNA as a probe. Sequence data of the *SART-1* are available from EMBL/GenBank/DDBJ under accession number AB006198. The difference of the sequence at nucleotide (nt) position 812 of the *ART-1* between PBMCs and KE4 was further analyzed by treatment of the PCR products with an SecI restriction enzyme. Amplification was performed for 30 cycles (1 min at 94°C, 2 min at 56°C, and 2 min at 72°C) with the primers of 5′-CCAAGTTACTGGAGGAGATGG-3′ (forward primer) and 5′-TTGGACAGGATAGAGCGAGG-3′ (reverse primer).

Preparation of Glutathion S-transferase Fusion Proteins and Rabbit Antisera. For SART-$1_{800/GST}$ (GST, glutathione s-transferase) protein, the full length of *SART-1* was digested with EcoRI and NotI at the multiple cloning site of pCMV-SPORT, and then ligated into the pGEX-4T-2 vector (Pharmacia Biotech AB, Uppsala, Sweden). For SART-$1_{6A1\text{-}1D7/GST}$ protein, the *SART-1* cDNA fragment (nt 1,663–2,449) was amplified by PCR using a forward primer 5′-TGGGAATTCGATGAGGATCCCGAGC-3′ (sf-1), and a reverse primer 5′-TACGGGCGGCCGCTGTCACTTGGT-3′ (sr-1). Amplified product was digested with EcoRI and NotI, and ligated to the pGEX-5X-3 (Pharmacia Biotech AB). For SART-$1_{67/GST}$ protein, the *SART-1* cDNA fragment (nt 1,663–1,866) was amplified by PCR using a sf-1 primer and a reverse primer 5′-CGTGAATTCTCACCGTGCTCCAGCC-3′. Amplified product was digested with EcoRI and ligated to the pGEX-5X-3. For SART-$1_{219/GST}$ protein, the *SART-1* cDNA fragment (1,781–2,449) was amplified by PCR using a forward primer 5′-GAGAATTCCATGGACTTTGAACGGGATG-3′ (sf-2) and a sr-1 primer. Amplified product was digested with EcoRI and NotI, and ligated to the pGEX-5X-3. Polyclonal anti–SART-$1_{800/GST}$, anti–SART-$1_{6A1\text{-}1D7/GST}$, anti–SART-$1_{67/GST}$, and anti–SART-$1_{219/GST}$ Abs were prepared by immunization of rabbits with purified SART-$1_{800/GST}$, SART-$1_{6A1\text{-}1D7/GST}$, SART-$1_{67/GST}$, and SART-$1_{219/GST}$ proteins, respectively, by the methods previously reported (19).

Preparation of SART-1–tag Fusion Protein in Expression Vector Constructs. For preparation of the *SART-*$1_{800/myc}$, the *SART-1* of positions 29–2,449 was amplified by PCR using a forward primer 5′-GCTCGGAATTCACGTGCCACTATGGG-3′ and a reverse primer 5′-AGGGAATTCTCGCTTGGTGATGGTGTTC-3′ (sr-2). Amplified product was digested with EcoRI, and ligated to pcDNA3.1/Myc-His vector (Invitrogen). The gene encoding a tag peptide was ligated to the 2,438 position before the stop codon of the third frame, which was used as the *SART-*$1_{800/myc}$. Similarly, the *SART-1* fragment of positions 1,663–2,449 or 1,782–2,449 was amplified by PCR using a sf-1 and a sr-2 primer or a sf-2 primer and a sr-2 primer, and the amplified product was used for preparation of the *SART-*$1_{6A1\text{-}1D7/myc}$ or *SART-*$1_{219/myc}$, respectively.

Western Blot Analysis. The samples were lysed with a buffer consisting of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.2 mM PMSF (Sigma Chemical Co., St. Louis, MO), and 0.03 one trypsin inhibitor unit (TIU)/ml aprotinin, sonicated, and centrifuged at 14,000 rpm for 20 min, and the supernatant was used as the cytosol fraction. Then, the pellet was lysed with a buffer consisting of 7.2 M urea, 1.6% Triton X-100, 0.8% dithiothreitol, and 2% lithium dodecyl sulfate, and was centrifuged, and the supernatant was used as the nuclear fraction. The lysate was separated by SDS-PAGE. The proteins in acrylamide gel were blotted to Hybond™-polyvinylidene difluoride (PVDF) membrane (Amersham) and were incubated with appropriate Abs for 4 h at room temperature. The other methods of Western blot analysis were previously described (19).

KE4 CTL, Its Sublines, and CTL Assay. HLA-A2601–restricted and SCC-specific CTL line (KE4 CTL) established from an esophageal cancer patient (18) was used in this study as effector cells for identification of the peptide antigens encoded by the *SART-1* gene. KE4 CTL sublines were established from the parental KE4 CTL line by the limiting dilution culture as reported (20). In brief, 1 cell/well (round bottomed 96-well microculture plate) of the parental KE4 CTL line was incubated with the culture medium (45% RPMI-1640 medium, 45% AIM-V® medium [GIBCO BRL], and 10% FCS [EQUITECH BIO, Ingram, TX] with 100 units/ml of IL-2 [Shionogi Pharm. Co., Osaka, Japan] and 0.1 mM MEM nonessential amino acids solution [GIBCO BRL]; termed as the culture medium) in the presence of irradiated (50 gray) allogenic PBMCs (2×10^5 cells/well) donated from three healthy volunteers as feeder cells. The proliferating CTL sublines were expanded in wells of 24-well microculture plates in the culture medium alone for up to 30 d. The sublines were tested for their cytotoxicity to the KE4 (A2402/2601), KE3 (A2402/A0201), and VA13 fibroblast cell lines in a 6-h ^{51}Cr–release assay as reported (18) at an E/T ratio of 5:1, and the 80 sublines showing cytotoxicity against the KE4, but not either KE3 or VA13, were used in this study.

Constructions of Deletion Mutants. The *pcDNA3/6A1-1D7* plasmid, a derivative of the pcDNA3 vector containing a 990-bp DNA fragment of the *6A1-1D7* gene corresponding to the nucleotide positions 1,517–2,506 of the *SART-1* gene and a CMV promoter for directing transcription, was digested with NotI for preparation of deletion mutants. The linear lysed DNA was subjected to the second restriction enzyme ApaI digestion to generate one end sensitive to ExoIII. ExoIII nuclease/Mung bean nuclease was performed according to the manufacturer's instructions (TaKaRa, Ootsu, Japan) to obtain five deletion mutants of the *6A1-1D7* (*6A1*$_{1\text{-}492}$ corresponding to nucleotide positions 1–492 of the *6A1-1D7* gene, *6A1*$_{1\text{-}625}$, *6A1*$_{1\text{-}736}$, *6A1*$_{1\text{-}839}$, and *6A1*$_{1\text{-}951}$). The *SART-*$1_{1\text{-}1,668}$ fragment was prepared by digestion of the *SART-1* in pSV-SPORT with the KpnI and BamHI, separated by agarose gel electrophoresis and purified by Qiaex gel extraction kit (Qiagen, Hilden, Germany). This fragment was ligated to the KpnI and BamHI sites of *6A1*$_{1\text{-}492}$, *6A1*$_{1\text{-}625}$, *6A1*$_{1\text{-}736}$, *6A1*$_{1\text{-}839}$, and *6A1*$_{1\text{-}951}$ in pcDNA3 vector, respectively, and five mutants (*SART-*$1_{1\text{-}2,008}$ corresponding to nucleotide positions 1–2,008, *SART-*$1_{1\text{-}2,141}$, *SART-*$1_{1\text{-}2,252}$, *SART-*$1_{1\text{-}2,353}$, and *SART-*$1_{1\text{-}2,467}$) were obtained. Further, the *SART-1* gene in pCMV-SPORT was digested with BamHI, ApaI, or SmaI, respectively, and each

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band was separated, purified, and ligated to prepare the three mutants (*SART-1*$_{1\text{-}793}$, *SART-1*$_{1\text{-}1,190}$, and *SART-1*$_{1\text{-}1,668}$).

Peptides and Assays. In this manuscript, amino acid (aa) positions were named based on the sequence of the predicted SART-1$_{800}$ protein because all the synthesized peptides are located in the region shared by both the SART-1$_{800}$ and SART-1$_{259}$ proteins that was translated in the third frame. A series of 22 different 10 mer, according to the predicted aa sequences corresponding to a part of deduced SART-1$_{800}$ protein (aa positions 730–800: SHRFHGKGSGKMKTERRMKKLDEEALLKKMSSSDTPLGTVALLQEKQKAQKTPYIVLSGSGKSMNANTITK), were prepared. Each peptide is a 10 mer that shares the same aa with the following peptide at positions 4–10. Six different nonapeptides were also prepared in which each of the three 10 mer (SART-1$_{736\text{-}745}$, SART-1$_{748\text{-}757}$, SART-1$_{784\text{-}793}$) was deleted at position 1 or 10. Further, each aa of the three nonapeptides (SART-1$_{736\text{-}744}$, SART-1$_{749\text{-}757}$, SART-1$_{785\text{-}793}$) was substituted by glycine (G) when it was not glycine or by threonine (T) when it was glycine to determine aa residues critical for binding to HLA-A2601 and CTL-mediated recognition. These peptides were purchased from Biologica (Nagoya, Japan). The purity was >70% in most of the peptides, and >95 % in those used for induction of CTLs. For detection of antigenic peptides, the HLA-A2601 or -A0201 cDNA (as a control) were transfected to the VA13 (2×10^4) cells and incubated for 5 h. Then, 200 μl of the RPMI-1640 medium containing 20% FCS was added and cultured for 2 d, followed by adding the peptides at the concentration of 10 μM in most experiments, or 10 nM to 50 μM in certain experiments. 2 h later, the supernatant was removed and the KE4 CTLs (10^4 cells/well) were added, incubated for 18 h, and 100 μl of supernatant was collected to measure IFN-γ by an ELISA kit in a duplicate assay.

Induction of CTL by Nonapeptides. PBMCs (2×10^6) from a KE4 patient that had been cryopreserved in a nitrogen tank were thawed in the morning of experiments, and were incubated with a nonapeptide (10 μM) in a well of a 24-well plate containing 2 ml of the culture medium. PBMCs from HLA-A2601$^+$ healthy volunteers were also used. At days 7 and 14 of culture, cells were collected, washed, and stimulated with antigen presenting cells consisting of the irradiated autologous PBMCs that had been preincubated with the same nonapeptide (10 μM) for 2 h followed by washing with PBS. The ratio of the responder to stimulator cell was 10:1. Cells were harvested at day 21 of the culture, and most of them were tested for their CTL activity in a 6-h ^{51}Cr-release assay. Some of them were provided for preparation of the CTL sublines by incubation of 10 cells/well (round bottomed 96-well microculture plate) with the culture medium in the presence of irradiated allogenic PBMCs as feeder cells. These cells from the microculture were tested for their activity at 10 d of culture to produce IFN-γ in response to tumor cells by an ELISA. Several sublines from well-proliferating wells were further expanded in the culture medium alone in wells of a 24-well plate, and were tested for their cytotoxicity to the KE4 and KE3 tumor cells at an E/T ratio of 5:1 in a 6-h ^{51}Cr-release assay at 15 d of the culture.

Results

Identification of the 6A1-1D7 Gene. The total of 10^5 cDNA clones from cDNA library of the KE4 tumor cells were tested for their ability to stimulate IFN-γ production by the KE4 CTLs after cotransfection with the HLA-A2601 into the VA13 human fibroblast cells. This method allows

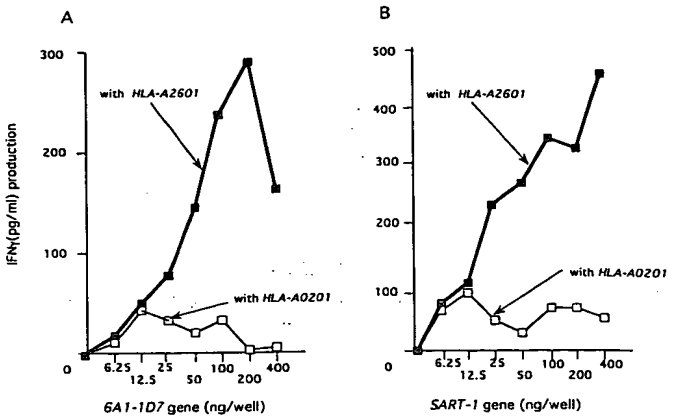

Figure 1. Recognition of the SART-1 gene products by the KE4 CTLs. Different amounts of the *6A1-1D7* (Fig. 1 *A*) or the *SART-1* cloned from the KE4 tumor (Fig. 1 *B*) and 100 ng of HLA-A2601 or HLA-A0201 cDNA were cotransfected into VA13 cells, followed by testing their ability to stimulate IFN-γ production by the KE4 CTLs. The background of IFN-γ production by the KE4 CTLs in response to VA13 cells (~200 pg/ml) was subtracted in the figure. Similar results were obtained in the *SART-1* cloned from the PBMCs (data not shown).

identification of genes encoding tumor-rejection antigens (1–6). After repeated experiments for the several candidate clones, one clone (*6A1-1D7*) was confirmed to encode a tumor antigen recognized by the KE4 CTLs when cotransfected with HLA-A2601 (Fig. 1 *A*). The sequence of this cDNA clone proved to be 990 bp long. Expression of this gene was investigated by Northern blot analysis. A band of ~2.6 kb was observed in all the normal tissues and tumor cell lines tested (Fig. 2). The relative level of mRNA expression was within the range of 2.3 ± 2.9 in all the samples except for testis (the expression level: 7.5) and pancreas (17.4) (Fig. 2). These results suggest that this gene was ubiquitously expressed at the mRNA level with higher expression in testis and pancreas, and that the 990-bp-long cDNA was incomplete.

Identification of the SART-1 Gene. A 2,506-bp-long gene was independently cloned from the cDNA libraries of KE4 tumor and PBMCs of healthy donors using the *6A1-1D7* as a probe (Fig. 3). The (nt) sequences of these clones were identical with the exception of the position 812 (cytosine in the KE4 versus thymine in PBMCs). This would be due to a genetic polymorphism, but not due to a point mutation, since the samples in which the nt position 812 was cytosine were the KE4 CTLs, a B cell line from the KE4 patient (Bec-1), fetal liver, COS cells, and 16 of 22 solid tumor cell lines tested, whereas it was thymine in testis, VA13 cells, and the other six tumor cell lines. An aa translated from these codons in the third frame is identical (ACC, ACU = threonine). KE4 CTLs also recognized VA13 cells cotransfected with these new genes and HLA-A2601 (Fig. 1 *B*). Both clones contained the *6A1-1D7* at positions 1,517–2,506. This 2,506-bp-long gene showed no similarity to known sequences, and was tentatively named as the *SART-1* gene. Although the *SART-1* mRNA was ubiquitously expressed, the KE4 CTLs did not recognize

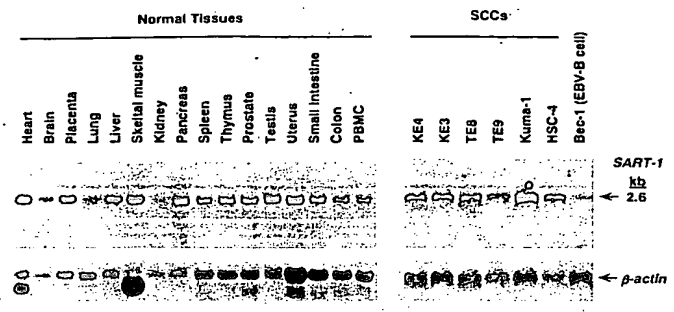

Figure 2. Expression of the *SART-1*. 21 tumor cell lines (KE4, KE3, TE8, Kuma-1, HSC4, QG56, Sq-1, A549, MKN28, Colo201, SW620, KMG-A, R-28, 86-2, LK79, LC65A, KIM-1, KYN-1, M36, M73, NALM-1; reference 18), PBMCs, Bec-1, COS, or 16 tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas on human multiple tissue Northern blot, and spleen, thymus, prostate, testis, uterus, small intestine, colon, and peripheral blood leukocyte on human multiple tissue Northern blot IV; Clontech Lab., Inc., Palo Alto, CA) were provided for Northern blot analysis with the *6A1-1D7* as a probe. Some of the results are shown in the figure.

nonmalignant cells including Bec-1 cells (18) or VA13 cells transfected with HLA-A2601 alone (Fig. 1). This might be due to preferential expression of tumor antigens on the malignant cells by the mechanism of posttranscriptional regulation.

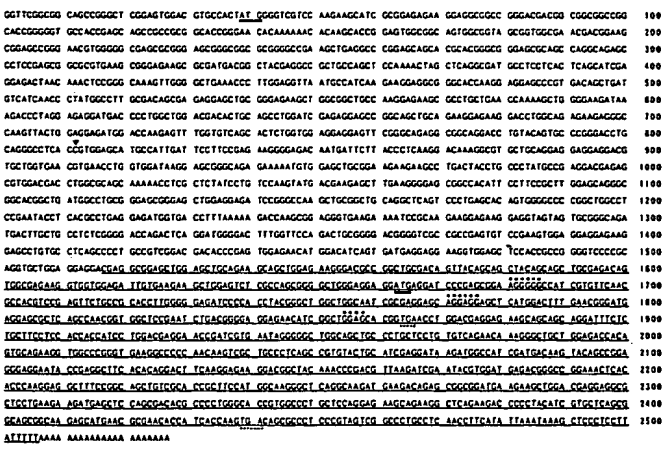

Figure 3. Nucleotide sequence of the *SART-1*. The cloned cDNA (*6A1-1D7*) was initially provided for the nucleotide sequencing. The sequence of *6A1-1D7* is 990 bp long (positions 1,517–2,506, *underlined by the solid line*) that has an ORF of 201 bp long encoding 67 aa if the first AUG codon (1,663–1,665, *underlined by the bold line*) and stop codon (1,864–1,866, *underlined by the dotted line*) in the first frame are used for protein synthesis. One S-D and each of the two different S-D–like sequences are marked by the dot on the top. The 2,506-bp-long *SART-1* was then cloned from cDNA libraries of the KE4 tumor and human PBMCs. The *SART-1* has an ORF 2,400 bp long encoding 800 aa when the first AUG codon (39–41, *underlined by the bold line*) and stop codon (2,439–2,441, *underlined by the dotted line*) are used for protein synthesis in the third frame. There was only one nt difference at the position 812 (marked by ∇) between the *SART-1* of KE4 tumor and PBMCs (cytosine in the KE4 tumor versus thymine in PBMCs). These sequence data are available from EMBL/GenBank/DDBJ under accession number AB006198.

We then intended to investigate the SART-1 protein expression in various cells and tissues by Western blot analysis with anti–SART-$1_{800/GST}$ and –SART-$1_{6A1\text{-}1D7/GST}$ Abs, since both the *SART-1* and *6A1-1D7* encoded a tumor antigen recognized by the KE4 CTLs. The first AUG codon resided at positions 39–41 of the *SART-1* in the third frame with suitable context (CCACUAUGG; Fig. 3) for initiation of protein synthesis (21, 22). The *SART-1* thus contains an open reading frame (ORF) of 2,400 bp encoding a protein of 800 aa residues (SART-1_{800}). In contrast, the first AUG codon of the *6A1-1D7* exists at positions 1,663–1,665 with unfavorable content (GGAGGAUGA) in the first frame. Between this first AUG and the stop UGA (1,864–1,866) codon of the *6A1-1D7*, there is one Shine-Dalgarno (S-D) sequence (AGGAGG, 1,771–1,776), one S-D–like sequence (AGGGGG, 1,681–1,686), and the other S-D like sequence (GGAG at seven different regions) that are known to induce frame shifting in prokaryotic mRNAs (23, 24). A protein of 259 aa (SART-1_{259}) could be translated if any of these S-D sequences induces −1 frame shifting and change the stop codon from the positions 1,864–1,866 of the first frame to the positions 2,439–2,441 of the third frame. If not, a peptide of 67 aa could be translated in the first frame.

Expression of the SART-1_{800} Protein. An Ab to the SART-$1_{800/GST}$ recognized a 125-kD band of SART-1_{800} protein after cleavage of GST with thrombin (data not shown), and recognized a 125-kD band in the nuclear fraction of PBMCs activated with 10 μg/ml of PHA (PHA blasts), KE4 tumor, and Bec-1, but not unstimulated PBMCs (Fig. 4 *A*). No protein in the cytosol was recognized by this Ab in any samples tested. The 125-kD band was also expressed in the nucleus of the majority of tumor tissues, tumor cell lines, and normal cell lines tested, but was not expressed in normal tissues except for testis and fetal liver. The summary is shown in Table 1. When the *SART-1* of positions 29–2,449 (*SART-1_{800}*) was transfected to VA13, intensities of the 125-kD band in both the nuclear and cytosol fractions increased (Fig. 4 *A*). Furthermore, this Ab and anti-myc monoclonal Ab recognized a 132-kD band of the VA13 cells transfected with the *SART-1* of positions 29–2,449 in conjunction with pcDNA3.1/Myc-His vector (*SART-$1_{800/myc}$*; Fig. 4 *A*). The different migration of these bands (125 and 132 kD) will be due to a tag peptide (theoretically ~5 kD). These results suggest that the 125 kD of the SART-1_{800} protein was expressed in the nucleus of proliferating cells including normal and malignant cells, but not in nonproliferating cells, nor any normal tissues except for testis and fetal liver.

Expression of the SART-1_{259} Protein. An Ab to the SART-$1_{6A1\text{-}1D7/GST}$ recognized a 43-kD band of the recombinant SART-$1_{6A1\text{-}1D7}$ protein after cleavage of GST with factor Xa (Fig. 4 *B*). Therefore, the SART-1_{259} could be translated by the mechanism of −1 frame shifting in the prokaryotic mRNA, and be recognized by anti-SART-$1_{6A1\text{-}1D7/GST}$ Ab. This Ab also recognized a 43-kD protein in the cytosol of KE4 and TE9 esophageal SCC cell lines, fresh esophageal SCCs, and lung SCCs and adenocarcinomas, but not PBMCs (Fig. 4 *B*). No protein in the nucleus

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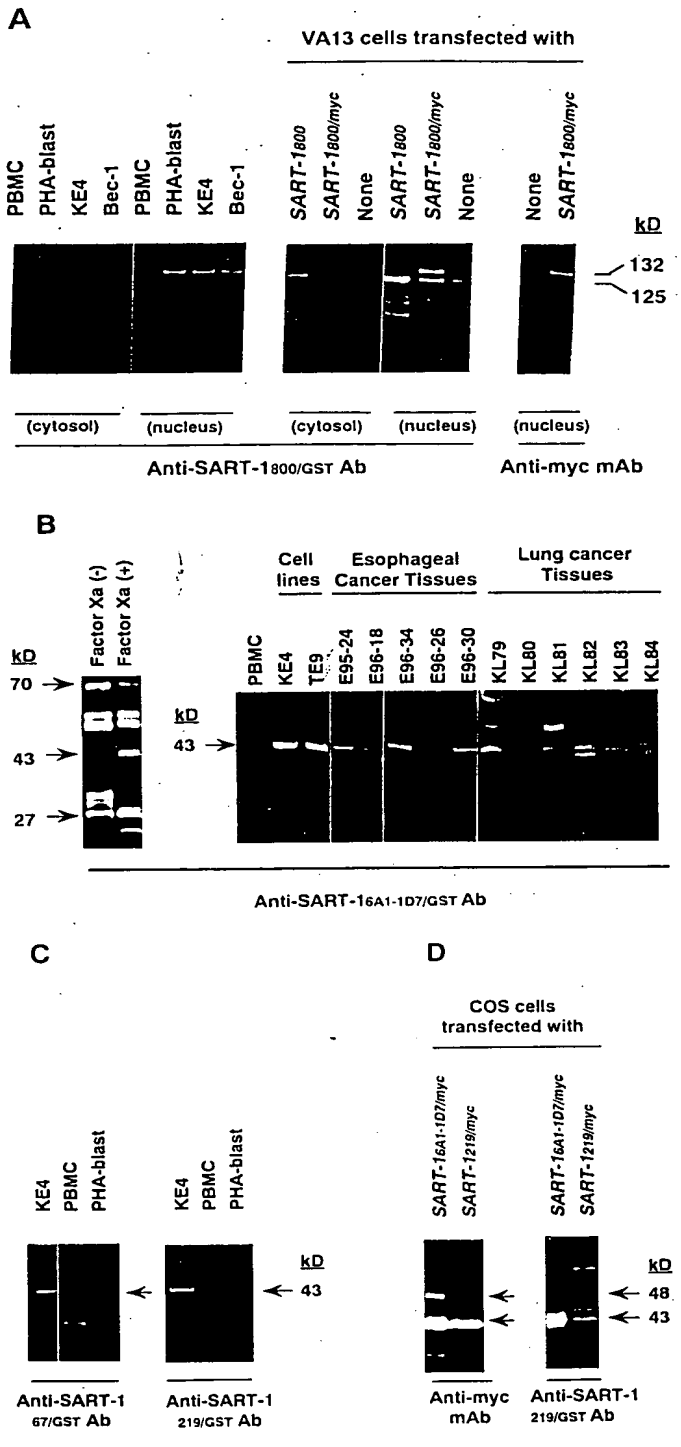

Figure 4. Expression of SART-1_{800} and SART-1_{259} proteins. Tumor cell lines used for Western blot analysis were head and neck SCCs (Ca9-22, HSC3, HSC4, Kuma-1, and Kuma-3), esophageal SCCs (KE4, KE3, TE8, TE9, TE10, and TE11), lung adenocarcinomas (1-87, LK87, PC-9, A549, 11-18, and RERF-LC-MS), lung SCCs (Sq-1, RERF-LC-AI, and QG56), leukemia cells (MOLT-4, HPB-ALL, HPB-MLT, HUT-102, BALL-1, NALM16, ARH77, THP1, U937, HL60, ML-1, ML-2, NALL-1, SPI-801, K562, and HEL), and melanomas (M36, M73) (18). PBMCs, PHA blasts, fibroblast cells (WI-38, VA13), and tumor tissues from various organs were also studied. (*A*) Expression of the SART-1_{800} protein was investigated by Western blot analysis with anti–SART-$1_{800/GST}$ Ab. Anti-myc monoclonal Ab (Invitrogen) was also used for analysis of VA13 transfected with the *SART-*$1_{800/myc}$. (*B*) Expression of the SART-1_{259} was investigated with anti–SART-$1_{6A1\text{-}1D7/GST}$ Ab. In the left gel, 70-, 43-, and 27-kD bands corresponded to the SART-$1_{6A1\text{-}1D7/GST}$, SART-$1_{6A1\text{-}1D7}$, and GST, respectively. The data of the cytosol fraction were shown. No bands were detected by this Ab in the nucleus of any samples tested (data not shown). (*C*) Expression of the SART-1_{259} was investigated with anti–SART-$1_{67/GST}$ and anti–SART-$1_{219/GST}$ Abs. The data of the cytosol fraction were shown. (*D*) Anti-myc and anti–SART-$1_{219/GST}$ Abs were used for analysis of COS cells transfected with the *SART-*$1_{6A1\text{-}1D7/myc}$ or *SART-*$1_{219/myc}$. The total lysate was used for experiments.

Table 1. *Expression of the SART-*1_{800} *and SART-*1_{259} *Proteins in Normal and Cancer Cells and Tissues*

	SART-1_{800} (nucleus)*		SART-1_{259} (cytosol)‡	
	Cell lines	Tissues	Cell lines	Tissues
Normal				
PBMC	0/5§	—	0/5	—
PHA blast	2/2	—	0/2	—
Fibroblast	2/2	—	0/2	—
Fetal liver	—	1/1	—	1/1
Newborn liver	—	0/1	—	0/1
Liver	—	0/1	—	0/1
Testis	—	1/1	—	3/3
Placenta	—	0/1	—	0/2
Esophagus	—	0/2	—	0/4
Pancreas	—	0/1	—	0/1
Cancer				
Head and neck SCC	2/2	2/2	3/5	7/7 (100%)
Esophageal SCC	5/5	3/5	4/6	18/30 (60%)
Lung cancer				
Adenocarcinoma	3/3	7/7	3/6	16/35 (47%)
SCC	2/2	3/4	3/3	8/17 (47%)
Leukemia	4/4	4/4	0/16	0/10 (0%)
Melanoma	1/1	—	0/2	0/10 (0%)

*Expression of the SART-1_{800} protein in the nucleus of various normal and cancer cells and tissues was investigated by Western blot analysis with anti–SART-$1_{800/GST}$ Ab.
‡Expression of the SART-1_{259} protein in the cytosol of various normal and cancer cells and tissues was investigated by Western blot analysis with anti–SART-$1_{6A1\text{-}1D7/GST}$ Ab.
§Number of positive per total samples tested are shown.

was detected by this Ab in any samples tested (data not shown). The 43-kD protein was expressed in the cytosol of all the head and neck SCC tissues tested, 60% of esophageal SCCs, and half of the lung SCCs and lung adenocarcinomas, but not observed in leukemia, melanomas, nor any normal tissues, normal cell lines, or normal cells except for fetal liver and testis (Table 1). These results suggest that the

SART-1_{259} protein was translated by the mechanism of −1 frame shifting using an internal ribosomal entry site in human mRNAs primarily from SCCs and adenocarcinomas, and was recognized by this Ab.

To investigate this possibility, we developed rabbit Abs against GST fusion protein with a peptide of 67 aa in the first frame (SART-$1_{67/GST}$) and a protein of 219 aa in the third frame (SART-$1_{219/GST}$), since each of the two proteins is necessary for construction of the SART-1_{259}. Both anti–SART-$1_{67/GST}$ and anti–SART-$1_{219/GST}$ Abs recognized a 43-kD band in the cytosol of the KE4, but not of PBMCs or PHA blasts (Fig. 4 *C*). These Abs also recognized a 43-kD protein of the other SCCs and lung adenocarcinomas, and the pattern of the reactivity was almost identical to that of anti–SART-$1_{6A1\text{-}1D7/GST}$ Ab shown in Table 1. The results suggest that this 43-kD protein consists of both a peptide of 67 aa in the first frame and a protein of 219 aa in the third frame. Furthermore, we prepared the plasmid construct in which the part of the *SART-1* at nt positions 1,663–2,449 or 1,782–2,449 was ligated into the pcDNA3.1/Myc-His vector (*SART-*$1_{6A1\text{-}1D7/myc}$ and *SART-*$1_{219/myc}$, respectively). When the *SART-*$1_{6A1\text{-}1D7/myc}$ was transfected to COS cells, two bands (48 and 43 kD) were detected with both anti-myc monoclonal and anti–SART-$1_{219/GST}$ Abs, whereas only a 43-kD band was detected in COS cells transfected with the *SART-*$1_{219/myc}$ (Fig. 4 *D*). The 48-kD protein might consist of 43 kD of SART-1_{259} plus 5 kD of a tag peptide that would be initiated by the AUG codon at positions 1,663–1,665 with the mechanism of −1 frame shifting. On the other hand, the 43-kD protein might consist of the 38-kD protein of the SART-1_{219} plus 5 kD of a tag peptide that would be initiated by the AUG codon at positions 1,782–1,784 in the third frame.

Identification of Regions Containing Antigenic Peptides for CTLs. To identify antigenic peptides encoded by the *SART-1* gene, we investigated the capability of deletion mutants of both the *6A1-1D7* and *SART-1* genes to stimulate IFN-γ production by the KE4 CTLs, since both genes encoded tumor antigens recognized by the KE-4 CTL as shown in Fig. 1. Higher levels of IFN-γ production were observed in the *6A1*$_{1\text{-}990}$ (full length), *6A1*$_{1\text{-}951}$, and *6A1*$_{1\text{-}839}$ when cotransfected with *HLA-A2601* into VA13 cells (Fig. 5 *A*). In contrast, no IFN-γ production was observed in the *6A1*$_{1\text{-}736}$ or any of the two mutants. Similarly, higher levels of IFN-γ production were observed in the *SART-*$1_{1\text{-}2,506}$ (full length), *SART-*$1_{1\text{-}2,467}$, and *SART-*$1_{1\text{-}2,355}$ (Fig. 5 *B*). In contrast, a very low level or no IFN-γ production was observed in the *SART-*$1_{1\text{-}2,252}$ or any of the other five mutants, respectively. These results suggest that antigenic peptide(s) mainly resided within the 254-bp region of the 3′ end of both the *6A1-1D7* and *SART-1*. This region encodes 62 deduced aa of the SART-1 protein at the positions of 737–800 in the third frame, which was shared by both the *6A1-1D7*–derived SART-1_{259} protein and the *SART-1*–derived SART-1_{800} protein.

Determination of Peptide Antigens. A series of 22 SART-1 oligopeptides (10 mer) corresponding to the region shown above were loaded to the VA13 cells that had been transfected with HLA-A2601 or -A0201, and tested for their ability both to stimulate IFN-γ production and to be recognized by the KE4 CTLs in a ^{51}Cr–release assay. Representative results are shown in Fig. 6 *A*. The three 10 mer (SART-$1_{736\text{-}745}$ [KGSGKMKTER], SART-$1_{748\text{-}757}$ [KKLDEEALLK], and SART-$1_{784\text{-}793}$ [IVLSGSGKSM]) possessed the activity to stimulate significant levels of IFN-γ production (>50 pg/ml), whereas none of the other 10 mer did. The SART-$1_{736\text{-}745}$ or SART-$1_{748\text{-}757}$ peptide had the high (45% lysis at an E/T ratio of 5:1) or low (10% lysis) activity to be recognized when loaded on VA13 cells transfected with *HLA-A2601*, respectively. None of the other 10 mer, including the SART-$1_{784\text{-}793}$, had the significant level (>10% lysis) of activity in a ^{51}Cr–release assay.

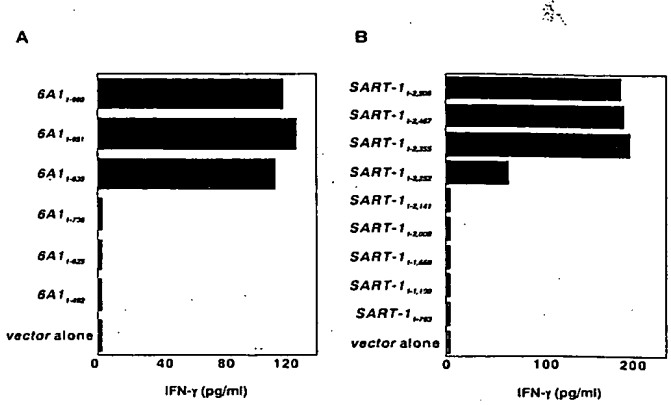

Figure 5. Identification of regions containing antigenic peptides for CTL. Deletion mutants of *6A1-1D7* gene (*6A1*$_{1\text{-}492}$, *6A1*$_{1\text{-}625}$, *6A1*$_{1\text{-}736}$, *6A1*$_{1\text{-}839}$, and *6A1*$_{1\text{-}951}$) and the full length of *6A1-1D7* in *A* or mutants of *SART-1* gene (*SART-*$1_{1\text{-}2,008}$, *SART-*$1_{1\text{-}2,141}$, *SART-*$1_{1\text{-}2,252}$, *SART-*$1_{1\text{-}2,355}$, and the others) and the full length of *SART-1* in *B* were cotransfected to VA13 cells (2×10^4) with HLA-A2601 or -A0201, and 2 d later these cells were tested for their ability to stimulate IFN-γ production by the KE4 CTLs. The background of IFN-γ production by the KE4 CTLs in response to VA13 cells transfected with both each mutant and HLA-A0201 (~50 pg/ml) was subtracted in the figure.

Six different nonapeptides from these three 10 mer with deletion of one aa at position 1 or 10 were tested for their ability to stimulate IFN-γ production by the parental KE4 CTL (Fig. 6 *B*). Each nonapeptide (SART-$1_{736\text{-}744}$ [KGSGKMKTE], SART-$1_{749\text{-}757}$ [KLDEEALLK], and SART-$1_{785\text{-}793}$ [VLSGSGKSM]) had higher activity to stimulate IFN-γ production than had the parental 10 mer. In contrast, each of the remaining nonapeptides failed to stimulate IFN-γ production.

To confirm the presence of a peptide-specific CTL, 80 KE4 CTL sublines were tested for their reactivity to each of the three 10 mer (SART-$1_{736\text{-}745}$, SART-$1_{748\text{-}757}$, and SART-$1_{784\text{-}793}$). 4, 5, or 6 of 80 of the KE4 CTL sublines showed the SART-$1_{736\text{-}745}$, SART-$1_{748\text{-}757}$, or SART-$1_{784\text{-}793}$ peptide–specific reactivity, respectively. The representative results are shown in Fig. 7.

In the SART-$1_{736\text{-}744}$ peptide, the ability to stimulate IFN-γ production was observed at 50 nM with the maximal level at 3 μM (Fig. 8). This ability was observed as low as 10 nM with the maximal level at 0.78 μM in the cases of both the SART-$1_{748\text{-}757}$ and SART-$1_{785\text{-}793}$ peptides.

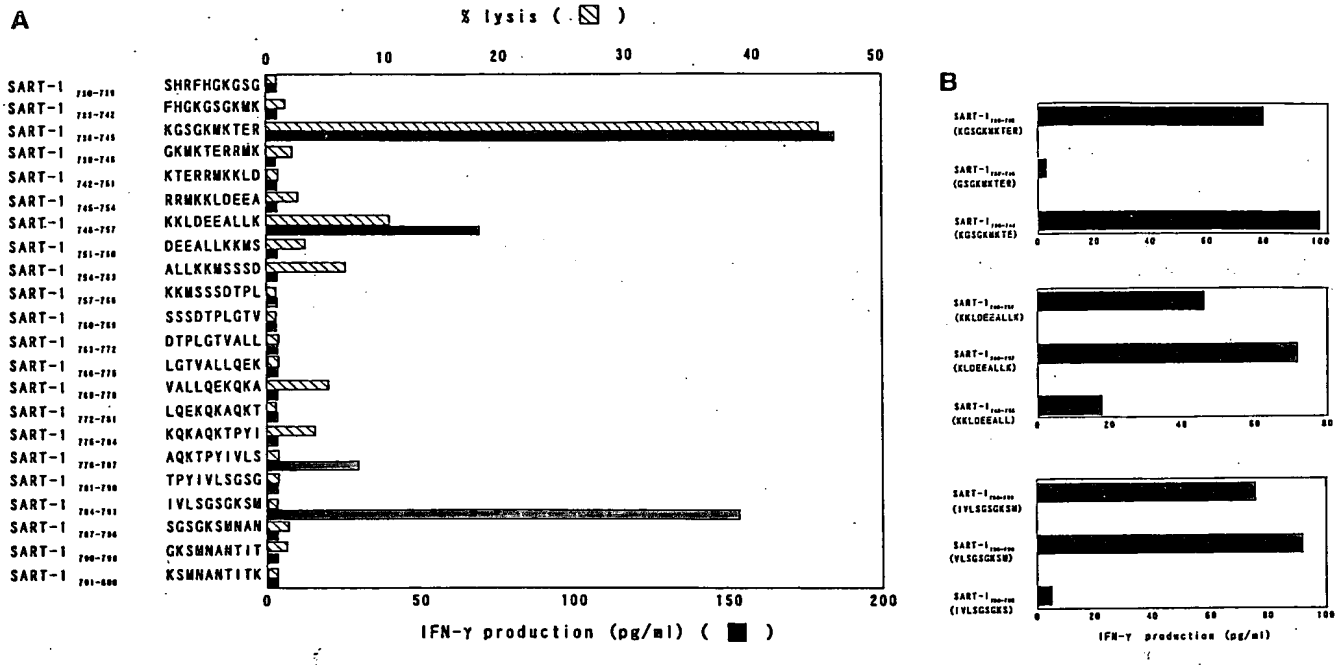

Figure 6. Determination of peptide antigens. A series of 22 SART-1 oligopeptides (10 mer; 10 μM) in *A* or 6 different nonapeptides (10 μM) from three 10 mer (SART-$1_{736-745}$, SART-$1_{748-757}$, and SART-$1_{784-793}$) with deletion of one aa at position 1 or 10 in *B* were loaded for 2 h to the VA13 cells (2×10^4) transfected with HLA-A2601 or -A0201. For IFN-γ production, the KE4 CTLs (10^4) were added, incubated for 18 h, and the culture supernatant was collected for measurement of IFN-γ by the ELISA in duplicate assays. The background of IFN-γ production by the KE4 CTLs in response to each peptide loaded to the VA13 cells transfected with HLA-A0201 (~50 pg/ml) was subtracted in the figure. In a ^{51}Cr–release assay, these VA13 cells were labeled with $Na_2\ ^{51}CrO_4$ for 1 h followed by adding the KE4 CTLs (5×10^4). 6 h later, the supernatant was harvested for measurement of the radioactivity in triplicate assays as reported (18). The background of percent lysis by the KE4 CTLs of the VA13 cells that were transfected with HLA-A0201 and loaded by each peptide was <5%.

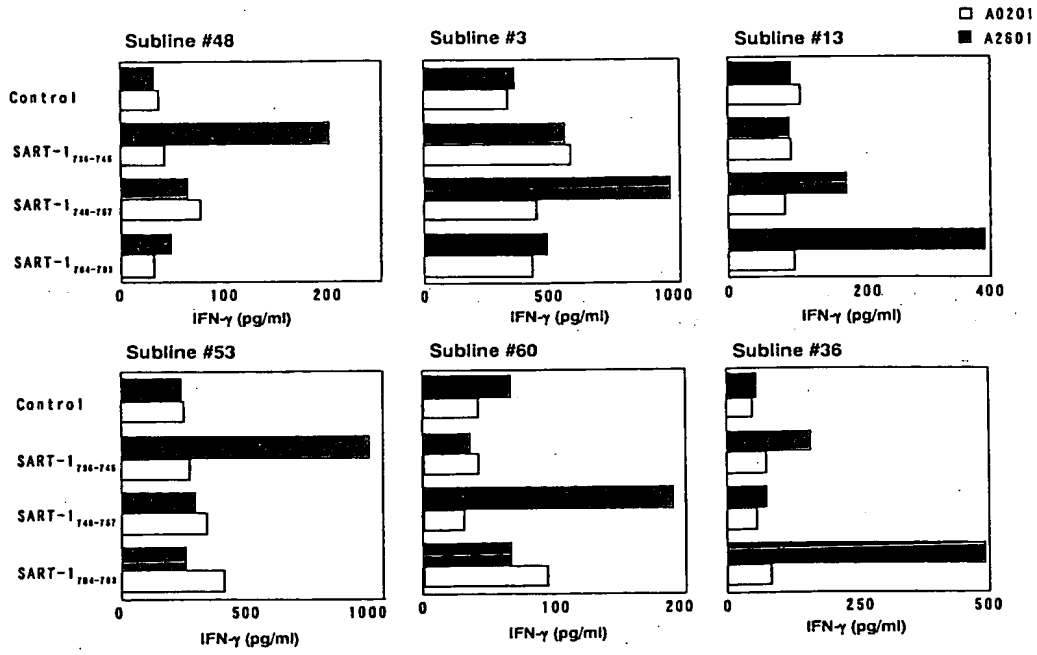

Figure 7. CTL sublines recognizing each nonapeptide. 80 KE4 CTL sublines were tested for their ability to produce IFN-γ by recognition of each of the three 10 mer (SART-$1_{736-745}$, SART-$1_{748-757}$, and SART-$1_{784-793}$) that was loaded at 10 μM on the VA13 cells for 2 h transfected with HLA-A2601 or -A0201. Detailed methods are shown in the legend for Fig. 6. Four, five, or six of 80 of the KE4 CTL sublines reacted to the SART-$1_{736-745}$, SART-$1_{748-757}$, or SART-$1_{784-793}$, respectively. The representative results from the peptide-specific CTL sublines (No. 48 and 53: the SART-$1_{736-745}$–specific CTLs; No. 3 and 60: the SART-$1_{748-757}$–specific CTLs; and No. 13 and 36: the SART-$1_{784-793}$–specific CTLs) are shown in the figure. The other CTL sublines were mostly not reactive to any of the 10 mer, and only a few sublines were reactive to two of the three 10 mer (data not shown). None of the sublines were reactive to all the three peptides.

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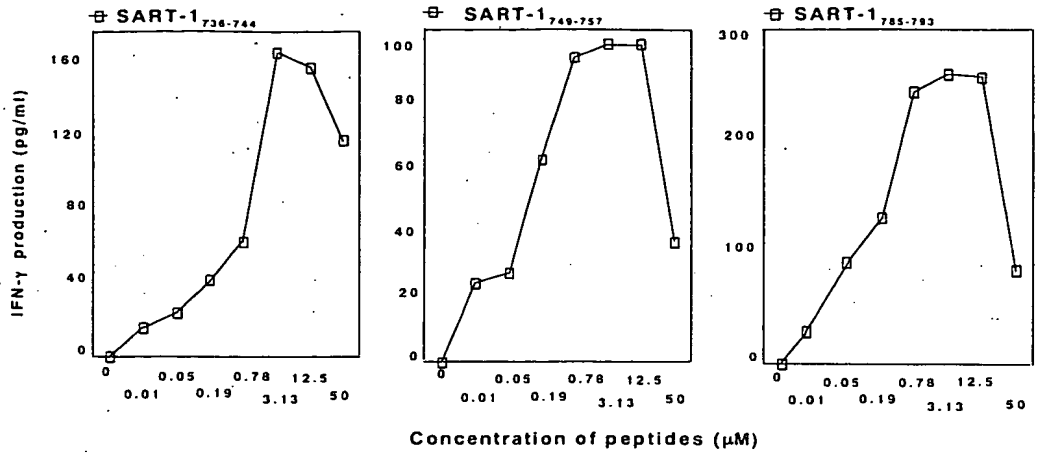

Figure 8. Dose dependency of nonapeptides. Various doses of each of the nonapeptides (SART-$1_{736-744}$, SART-$1_{749-757}$, and SART-$1_{785-793}$) were loaded for 2 h on VA13 cells transfected with HLA-A2601 or -A0201 followed by testing their ability to stimulate IFN-γ production by the parental KE4 CTLs. Detailed methods are shown in the legend for Fig. 6.

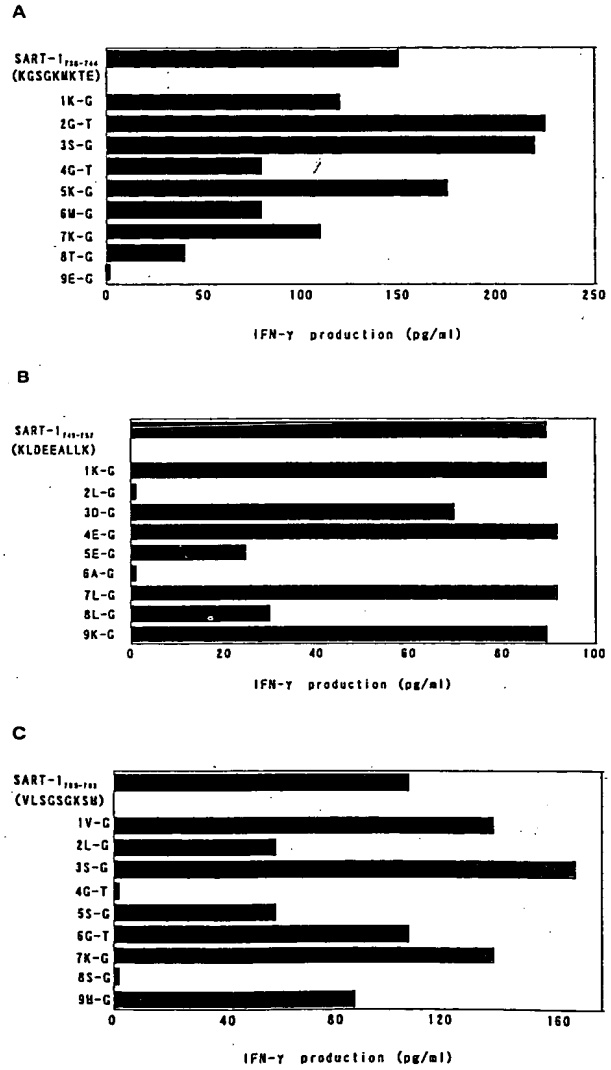

Figure 9. Determination of aa required for CTL-mediated recognition. Each aa of the three nonapeptides (SART-$1_{736-744}$, SART-$1_{749-757}$, and SART-$1_{785-793}$) was substituted by glycine when it was not glycine, or threonine when it was glycine. These substituents along with the parental nonapeptides (10 μM) were loaded for 2 h to the VA13 cells transfected with HLA-A2601 or -A0201 followed by testing their ability to stimulate IFN-γ production by the parental KE4 CTLs. Detailed methods are shown in the legend for Fig. 6.

Determination of aa Required for CTL-mediated Recognition. Each aa of the three nonapeptides (SART-$1_{736-744}$, SART-$1_{749-757}$, and SART-$1_{785-793}$) was substituted by glycine (G) when it was not glycine or by threonine when it was glycine. These peptides, along with the parental nonapeptides, were tested for their ability to stimulate IFN-γ production by the parental KE4 CTLs. The representative results are shown in Fig. 9. The ability of the SART-$1_{736-744}$ disappeared or extremely decreased when glutamic acid (E) or threonine (T) at position 9 (*9E-G* in Fig. 9) or 8 (*8T-G* in Fig. 9) was substituted, respectively. It also decreased when glycine or methionine (M) at position 4 or 6 was substituted, whereas it slightly increased when glycine or serine (S) at position 2 or 3 was substituted to threonine or glycine, respectively. The ability of SART-$1_{749-757}$ disappeared when leucine (L) or alanine (A) at position 2 or 6 was substituted. It also decreased when glutamic acid or leucine at position 5 or 8 was substituted. In a case of the SART-$1_{785-793}$, the ability disappeared when glycine or serine at position 4 or 8 was substituted. It decreased when leucine or serine at position 2 or 5 was substituted, whereas slightly increased when serine at position 3 was substituted.

Induction of CTLs by the Nonapeptides. The three nonapeptides (SART-$1_{736-744}$, SART-$1_{749-757}$, and SART-$1_{785-793}$) were tested for their ability to induce the CTLs against the autologous tumor cells from PBMCs of a KE4 patient. PBMCs stimulated with the SART-$1_{736-744}$ and their subline No. 1 showed higher levels of the KE4 autologous tumor cell lysis than those of the KE3 allogenic tumor cell lysis (Table 2). In contrast, PBMCs cultured with IL-2 alone or stimulated with SART-$1_{749-757}$ or SART-$1_{785-793}$ equally lysed both the tumor cells. The cells from the total of 144 microcultures (48 microcultures from PBMCs alone, 48 stimulated with the SART-$1_{736-744}$, and 48 with the

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Table 2. *Cytotoxicity in PBMCs Stimulated with the Three Nonapeptides**

Effector cells		Percent specific lysis (E/T ratio of 5:1) KE3 (A2402/ A0201)	KE4 (A2601/ A2402)
PBMC alone	Bulk culture	11.50	12.10
PBMC stimulated	Bulk culture	15.80	28.50
with SART-$1_{736-744}$	Subline No. 1	0.00	12.00
PBMC stimulated	Bulk culture	26.10	27.50
with SART-$1_{749-757}$	Subline No. 1	0.00	0.00
PBMC stimulated	Bulk culture	12.00	13.00
with SART-$1_{785-793}$			

*The three nonapeptides (SART-$1_{736-744}$, SART-$1_{749-757}$, and SART-$1_{785-793}$) were tested for their ability to induce CTLs against the KE4 tumor cells from PBMCs of a KE4 patient. After the stimulation, PBMCs or the sublines were tested for their cytotoxicity against the autologous KE4 and allogenic KE3 tumor cells at an E/T ratio of 5:1 in triplicate determinants in a 6-h ^{51}Cr–release assay. The mean values of percent specific lysis are shown in the table.

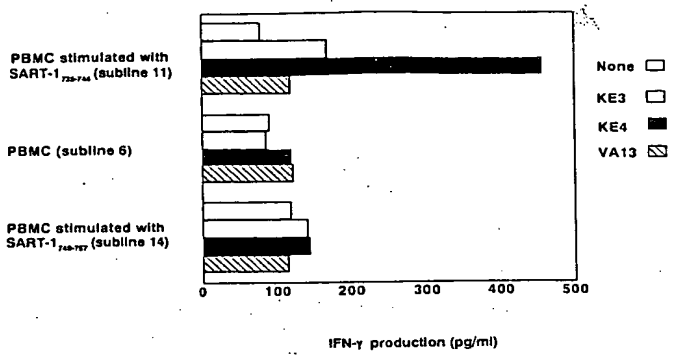

Figure 10. Induction of CTLs by the nonapeptides. The cells from total of 144 microcultures (48 microcultures from PBMCs alone, 48 stimulated with the SART-$1_{736-744}$, and 48 with the SART-$1_{749-747}$) were independently tested for their activity to produce IFN-γ in response to the KE3, KE4 tumor, and VA13 cells. The cells from 10 of 48 of the microcultures from the PBMCs stimulated with the SART-$1_{736-744}$ produced IFN-γ by recognition of the KE4, but not the other cells. The representative result of one microculture (*subline 11*) showing positive IFN-γ production is shown in the figure. In contrast, the cells from none of 48 of the microcultures besides one from either PBMCs alone or PBMCs stimulated with the SART-$1_{749-757}$ produced IFN-γ by recognition of the KE4 cells. The representative result of one microculture (subline 6 from PBMCs alone or subline 14 from PBMCs with the SART-$1_{749-757}$) showing negative IFN-γ production is shown in the figure.

SART-$1_{749-757}$) were independently tested for their activity to produce IFN-γ by recognition of the KE3, KE4 tumor, and VA13 cells. The cells from 10 of 48 of the microcultures from the PBMCs stimulated with the SART-$1_{736-744}$ produced higher levels of IFN-γ by recognition of the KE4, but not the other cells. The representative result of the one microculture showing positive IFN-γ production is shown in Fig. 10. In contrast, the cells from none of 48 of the microcultures besides one from PBMCs alone or PBMCs stimulated with the SART-$1_{749-757}$ produced higher IFN-γ by recognition of the KE4 tumor cells. The representative result of one microculture showing negative IFN-γ production is shown in Fig. 10.

Discussion

The results of this study suggest that *SART-1* gene is a bicistronic gene encoding two proteins, SART-1_{800} (125 kD) in the nucleus and SART-1_{259} (43 kD) in the cytosol. Most eukaryotic mRNAs have a single ORF and a single functional initiation site, which is usually the AUG codon that lies closest to the 5′ end (21, 22). However, there are some viral mRNAs that break this rule; two proteins are translated from either the same or a different ORF (25–27). Several human genes are also suggested to be bicistronic. liver-enriched transcriptional-activator protein (LAP) mRNA was found to be translated into two proteins, LAP and the liver-enriched transcriptional-inhibitory protein (LIP; 28, 29). The LIP contains the DNA-binding and dimerization domains, but is devoid of the transcription-activation domain. LAP and LIP seem to exhibit antagonistic activities. Another example is the glycoprotein (*gp*) *75* encoding two different polypeptides, gp75 recognized by sera from cancer patients and a peptide with 24 aa recognized by CTLs (30). However, the mechanism of posttranscriptional regulation in human mRNAs is scarcely understood at the present time. Therefore, *SART-1* shall be a novel tool to explore the mechanism.

It is of note that *SART-1* encodes a leucine zipper motif around nt positions 1,125–1,202 in the third frame (corresponding peptide: RELEEIRAKLRLQAQSLSTVGPRLAS). The leucine zipper motif is known to form homo- or heterodimers that can bind DNA and modulate transcription of many genes (31, 32). Indeed, the SART-1 gene product bound to DNA (our unpublished results). Although its biological functions are currently unknown, the SART-1_{800} protein might be involved in regulation of gene transcription, because it was localized in the nucleus of proliferating cells, possessed a leucine zipper motif, and bound to DNA. In contrast, the SART-1_{259} protein without leucine zipper motif expressed in the cytosol of SCCs and adenocarcinomas might inhibit the activity of the SART-1_{800}. If this is the case, these proteins might be involved in regulation of proliferation of epithelial cells and their malignant transformation.

The region of antigenic peptides encoded by the *6A1-1D7* and *SART-1* genes was 62 aa from the COOH terminus shared by the SART-1_{259} and SART-1_{800}. Therefore, both proteins could be used for antigen processing to present the antigenic peptides on the groove of the HLA-A2601 molecule, although the SART-1_{259} protein, but not the SART-1_{800}, is expected to be used as a major source of the antigenic peptides recognized by the KE4 CTL because of its preferential expression in the cytosol of tumor cells.

The three 10 mer and their nonapeptides in the region of SART-1 protein at positions of 730–800 were identified by an IFN-γ assay as antigenic peptides recognized by the HLA-A2601–restricted KE4 CTLs. Because of the presence of CTL sublines reacting to each of the three 10 mer among the 80 sublines tested, the parental KE4 CTL line would consist of the mixtures of these peptide-specific CTL clones. The other sublines were either not reactive to any of the 10 mer or reactive to two of the three 10 mer. Among these 10 mer, SART-$1_{736-745}$, and also SART-$1_{748-757}$ to some extent, but not SART-$1_{785-794}$, had the activity in a ^{51}Cr–release assay. SART-$1_{736-744}$, but not the others, possessed the ability to induce CTLs in PBMCs against the autologous tumor cells. Although the molecular basis for this discrepancy is presently unclear, SART-$1_{736-744}$ might be naturally expressed on the HLA-A2601 allele of the KE4 tumor cells.

Our results suggest that threonine and glutamic acid at positions 8 and 9 of SART-$1_{736-744}$ (KGSGKMKTE), leucine and alanine at positions 2 and 6 of SART-$1_{749-757}$ (KLDEEALLK), and glycine and serine at positions 4 and 8 of SART-$1_{785-793}$ (VLSGSGKSM) are critical for the recognition of each peptide by the parental KE4 CTLs. In addition, glycine and methionine at positions 4 and 6 of SART-$1_{736-744}$, glutamic acid and leucine at positions 5 and 8 of SART-$1_{749-757}$, and leucine and serine at positions 2 and 5 of SART-$1_{785-793}$ are important for the recognition. The binding motif for HLA-A2601 has not been determined as far as we know, and the KE4 CTL did not react to HLA-A2603$^+$ SCC and thus seemed to be HLA-A2601 restricted (18). The F pocket residues of these two subtypes are different (33), and therefore a binding motif at position 9 for them may be different from each other. Subsequently, it is difficult to compare our results of aa residues at position 9 to others, showing that valine or a hydrophobic residue at position 9 is important for binding to HLA-A26 (34, 35). With regard to the position 2, threonine, leucine, or valine was reported as the motif for binding to HLA-A26. Our results indicated that leucine of both SART-$1_{749-757}$ and SART-$1_{785-793}$ was required for binding to HLA-A2601 allele, and substitution of glycine to threonine at position 2 of the SART-$1_{736-744}$ rather increased its activity to induce IFN-γ production by the parental KE4 CTLs. From the results of the experiments of dose-dependent reactions, both SART-$1_{749-757}$ and SART-$1_{785-793}$ seemed to have higher affinity for binding to the groove of HLA-A2601 molecule than that of SART-$1_{736-744}$. Modified gp100 nonapeptides are reported to be more potent for induction from PBMCs of HLA-A2–restricted CTLs cytotoxic to melanoma cells (36). Therefore, a modified SART-$1_{736-744}$ peptide at position 2 from glycine to threonine, or probably to leucine or valine, may increase affinity of the binding to HLA-A2601, which in turn increase the ability to induce CTLs restricted to HLA-A2601$^+$ SCCs. This issue needs to be tested for development of better cancer vaccines.

The SART-$1_{736-744}$ peptide, but not the others, induced from the patient's PBMCs the CTLs restricted to the autologous tumor cells. CTL precursors in the patient's PBMCs increased by 10-fold after three rounds of stimulation with the peptide in vitro. This peptide failed to induce CTLs in PBMCs from any of three HLA-A2601$^+$ healthy donors tested under the conditions used in this study (data not shown). The SART-1_{259} protein was expressed in the cytosol of the majority of SCCs tested and half of lung adenocarcinomas. Because of its preferential expression in the cytosol of SCCs and adenocarcinomas, the SART-1_{259} protein, but not the SART-1_{800}, could be a major source of antigenic peptides recognized by CTLs. The HLA-A26 allele is found in ~22% of Japanese, 17% of Caucasians, and 14% of Africans (37). The A2601 subtype is found most frequently among the A26 subtypes (38). Therefore, the SART-1_{259} protein along with the SART-$1_{736-744}$ peptide could be useful for specific immunotherapy of relatively large numbers of HLA-A2601 patients with SCCs or adenocarcinomas as a cancer vaccine and also an antigen in vitro to induce CTLs for adoptive cellular therapy.

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